U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: FIND=1

U S APPLICATION NO (If known,see 37 CFR 1 5): 10/088236

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY CLAIMED
PCT/DK00/00522	20 September 2000	21 September 1999

TITLE OF INVENTION: METHOD FOR MATURATION OF CONIFER SOMATIC EMBRYOS

APPLICANT(S) FOR DO/EO/US: **Jens Iver FIND**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S C. 371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not transmitted by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a [ ] are transmitted herewith (required only if not transmitted by the International Bureau)
   b. [ ] have been communicated by the International Bureau
   c [ ] have not been made, however, the time limit for making such amendments has NOT expired.
   d [X] have not been made and will not be made
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U S.C 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information.
    [X] Courtesy copy of the International Application as filed.
    [X] Courtesy copy of the first page of the International Publication (WO 01/20972).
    [X] Courtesy copy of the International Preliminary Examination Report. There were no annexes.
    [X] Formal drawings, 9 sheets, Figures 1-9.
    [X] Courtesy Copy of the International Search Report.
    [X] Application Data Sheet

[X] The application is (or will be) assigned to: Woody Plant Biotech ApS,, whose address is Lundbygaardsvej 100, 4750 Lundby, Denmark.

JC13 Rec'd PCT/PTO 19 MAR 2002

U.S. APPLICATION NO (If known, see 37 CFR 1 5)	International Application No	Attorney's Docket No
10/088236	PCT/DK00/00522	FIND=1

17. [xx] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... ... **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... . ..... **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... . .. . **$100.00**

	CALCULATIONS PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1 492(e))	$ 130.00

Claims as Originally Presented	Number Filed	Number Extra	Rate	
Total Claims	28 - 20	8	X $18 00	$ 144.00
Independent Claims	1 - 3		X $84.00	$
Multiple Dependent Claims (if applicable)			+$280.00	$
TOTAL OF ABOVE CALCULATIONS =				$1,164.00

Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate	
Total Claims	- 20		X $18.00	$
Independent Claims	- 3		X $84 00	$
TOTAL OF ABOVE CALCULATIONS =				$1,164.00
Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.				$
SUBTOTAL =				$1,164.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$1,164.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3 31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$1,164.00
			Amount to be: refunded	$
			charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.

b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of $ 1,164 00, is attached.

c. [ ] Please charge my Deposit Account No. **02-4035** in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

d. **[XX]** The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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Date of this submission: **March 19, 2002**

SIGNATURE

Iver P. Cooper
NAME

28,005
REGISTRATION NUMBER

10/088236

#5/B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Jens Iver FIND	)	Art Unit:
IA No.: PCT/DK00/00522	)	
IA Filed: September 20, 2000	)	Washington, D.C.
For: METHOD FOR MATURATION...	)	May 17, 2002 Docket No.: FIND=1

SECOND PRELIMINARY AMENDMENT

Honorable Commissioner for Patents and Trademarks
Washington, D.C. 20231

Sir:

On page 1, immediately after the title, please insert he following new paragraph:

--This application is a 371 of PCT/DK00/00522, filed September 20,2000, published in English, which is a non-provisional of 60/161,938, filed October 28, 1999.--

REMARKS

This amendment is made to comply with 35 US§§119(e) and 120.

Respectfully submitted,
BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

By: _____
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Jens Iver FIND	)	Art Unit:
IA No.: PCT/DK00/00522	)	
IA Filed: September 20, 2000	)	Washington, D.C.
For: METHOD FOR MATURATION...	)	March 19, 2002 Docket No.: FIND=1

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents and Trademarks
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE CLAIMS

Please cancel claims 11, 13, 23-26, 29, 30, 34, 35 and 37.

Please amend the claims as follows:

7 (Amended). A method according to claim 1, whereby the culture medium in at least one of the steps further comprises a maturation agent.

10 (Amended). A method according to claim 7, whereby the maturation agent is abscisic acid at a concentration of between 0.1 and 200 uM.

18(Amended). A method according to claim 1, whereby the conifer is an Abies sp.

19 (Amended). A method according to claim 1, whereby the conifer is a Picea sp.

20 (Amended). A method according to claim 1, whereby the conifer is an Abies sp and the anti-auxin is PCIB at a concentration between 1 and 100uM.

27 (Amended). A method according to claim 3, whereby the embryogenic cell mass is cultured with a culture medium comprising a carbohydrate source.

28 (Amended). A method according to claim 27, where the embryogenic cell mass is cultured with a culture medium comprising sucrose, fructose, or glucose.

33 (Amended). A mature conifer somatic embryo produced by the method according to claim 1 having a water content less than 70%.

Please add the following new claims:

38. (New) A method according to claim 1 where the conifer is *Abies nordmannia*.

39. (New) A method according to claim 1 where the conifer is *Picea abies* or *Pichea sitchenis*.

REMARKS

Claims 1-10, 12-22, 27-28, 31-33, 36 and 38-39 presently appear in this case. The above amendments to the claims are being made in order to place this case in better condition for examination. Please enter this amendment prior to calculation of the filing fee in this case.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with Markings to Show Changes Made**."

Favorable consideration is earnestly solicited.

Respectfully submitted,
BROWDY AND NEIMARK, P.L.L.C.
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F:\,H\hoib\Findl\PTO\Preliminary Amendment.doc

VERSION WITH MARKINGS TO SHOW CHANGES MADE

7 (Amended). A method according to ~~any of the preceding claims~~claim 1, whereby the culture medium in at least one of the steps further comprises a maturation agent.

10 (Amended). A method according to ~~any of the claims 7 to 9~~claim 7, whereby the maturation agent is abscisic acid at a concentration of between 0.1 and 200 uM.

18(Amended). A method according to claim 1, whereby the conifer is an Abies sp ~~such as Abies nordmanniana~~.

19 (Amended). A method according to claim 1, whereby the conifer is a Picea sp ~~such as Picea abies or Picea sitchensis~~.

20 (Amended). A method according to claim 1, whereby the conifer is an Abies sp ~~such as Abies nordmanniana~~ and the anti-auxin is PCIB at a concentration between 1 and 100uM.

27 (Amended). A method according to claim 3, whereby the embryogenic cell mass is ~~being further~~ cultured with a culture medium comprising a carbohydrate ~~sources~~source.

28 (Amended). A method according to claim 27, where~~whereby~~ the embryogenic cell mass is ~~being further~~ cultured with a culture medium comprising sucrose, fructose, or glucose.

33 (Amended). A mature conifer somatic embryo produced by the method according to ~~any of the preceding claims~~claim 1 having a water content less than 70%.

Claims 11, 13, 23-26, 29, 30, 34, 35 and 37 have been cancelled.

Claims 38 and 39 have been added.

Method for maturation of conifer somatic embryos.

Technical field:

The invention relates to the field of methods for plant propagation by tissue culture techniques, more particularly for plant propagation of coniferous trees by somatic embryogenesis.

Prior art

Due to the very long generation cycles in conifers in general, breeding through crossing and selection proceeds very slowly and due to the fact that conifers are notorious outbreeders the offspring of even highly selected individuals varies enormously. However, through clonal propagation, it is possible to capture both the additive and the non-additive variation within a population whereby additional genetic gain is obtained. For a number of economically important conifers, methods have been developed for clonal propagation mainly as rooted cuttings, but for the vast majority of these species, sexual propagation via seeds is the only or the only cost-effective method for propagation.

Even in the cases, where methods for propagation via cuttings have been developed, the outcome is often not satisfactory, since rooting percentage declines with the age of the mothertree, and since there is a tendency for plagiotropic growth of cuttings not taken from the apical shoot. For these reasons there is a tremendous world-wide interest in developing cost-effective methods for reproducible clonal propagation of conifers.

The advent of genetic transformation as a tool for breeding of trees also necessitates the development of efficient and reproducible methods for regeneration of plants from transformed tissue. It is imperative that the regeneration methods are applicable to almost all cell lines in order to avoid any unintentional selection during the propagation step succeeding the transformation step.

In short, plant regeneration through somatic embryogenesis in conifers consists of a number of consecutive steps. First, an embryogenic culture is initiated from an ex-

plant, which could be either an embryo, mature or immature, a seedling, or recently also buds from adult trees (WO 99/23874 AFOCEL). This step is carried out on any suitable plant culture medium containing various plant growth regulators largely depending on the genus of the species in question. Typically, both auxin and cytokinin are included, but there are also reports on initiation using only cytokinin (Nørgaard & Krogstrup 1995, p 344, Table 1) or even initiation without any plant growth regulators (US 5,565,355 NEW ZEALAND FOREST RESEARCH INSTITUTE, Nørgaard & Krogstrup 1995, page 345).

For continued proliferation, the initiated cultures are either subcultured on medium with the same composition as the induction medium or they are subcultured on medium with lower concentrations of plant growth regulators. At this stage, the proliferating cell masses consist of more or less well differentiated immature somatic embryos, which morphologically correspond to a stage found in the developing seed in the early phase of seed development. Under optimal conditions, the somatic embryos do not undergo any further development during proliferation and mature embryos are thus not formed during this phase.

In order to obtain embryo maturation, the cultures need to be transferred to a plant culture medium, where – typically – auxin and cytokinin are omitted and abscisic acid (ABA) is included. In some cases, a short (1-2 weeks) transition step is included, during which the cell masses are cultured on plant culture medium devoid of plant growth regulators and sometimes including activated charcoal. This phase is believed to facilitate subsequent maturation, due to the lower content or absence of auxin and cytokinin in the culture medium and their possible removal by activated charcoal. A doubling of the subsequent maturation frequency has been reported (WO93/11660).

A number of factors have been shown to have a general stimulatory effect on the frequency of embryo maturation and/or on the quality of the mature embryos formed. The most important factor is the naturally occurring plant growth regulator abscisic acid. Today, this compound or analogues or derivatives thereof are included in almost all protocols for maturation of conifer somatic embryos.

Another factor of importance for the maturation process is the osmolality of the plant culture medium (WO 93/11660 UNIVERSITY OF SASKATCHEWAN). Increasing this by adding a non-permeating osmoticum such as PEG-4000 (Polyethyleneglycol-4000) has been shown to improve especially the quality of the mature embryos. The improvement is suspected to be caused by an increased level of triacylglycerides in the mature embryos. Triacylglycerides are deposited in the cells during maturation of zygotic embryos and are used as an energy source for the germination. PEG-4000 or similar compounds are routinely incorporated into maturation media.

In the vast majority of conifers, sucrose is used as the sole carbohydrate source for the maturation step. However, there are reports that especially maltose may give superior results. This has been reported for *Pinus spp* (US 5187092 INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY) and for *Abies nordmanniana* (Plant Science vol 124:211-221, NØRGAARD).

There are also reports that the inclusion of an auxin into the maturation medium, may stimulate both the number of mature somatic embryos formed, and their quality. US 5187092 INSTITUTE OF PAPER SCIENCE AND TECHNOLOGY discloses the use of 0.5-2.0 µM IBA + 10-40 µM ABA for the maturation of *Pinus taeda and Pseudotsuga menziesii* somatic embryos and Roberts et al (1990) discloses the use of 0.1-10 µM IBA + 40 µM ABA for the maturation of *Picea glaucaxengelmanii*. Thus, it has been shown for very diverse species from three different genera, that the inclusion of an auxin during maturation improves the process.

As pointed out in WO 96/37096 (CARTER HOLT HARVEY LTD.) the percentage of initiated cell lines that are able to form mature embryos is typically 1 – 10 %. WO 96/37096 mentions percentages up to 25 % obtained with selected embryogenic cell lines. If these methods are to be used for mass propagation and be combined with breeding in forestry or horticulture, it is of utmost importance that essentially no or very limited selection takes place in the propagation step. Valuable clones may be lost, if it is not possible to propagate them.

In *Picea abies* it has been possible to divide cell lines into two groups based on their morphology and the ability of the cell lines to produce mature somatic embryos. Cell lines capable of producing mature somatic embryos are termed A-type cell lines and

they readily produce mature embryos when subjected to known maturation methods with abscisic acid. On the other hand, approximately 50 % of all cell lines can be characterised as B-type cell lines, which do not readily produce mature somatic embryos (von Arnold et al 1995).

Another problem associated with the methods of the prior art is the lack of reproducibility.

In many species, proliferation during the maturation phase is a problem. When proliferation continues during maturation, the latter process is inhibited and in many cases the maturing embryos are overgrown by proliferating tissue. In the species belonging to *Picea*, *Pinus* and *Larix*, proliferation does not continue to the same extent as in species belonging to *Abies*, but may still constitute a problem. Maturing embryos are primarily formed at the edge of the cell mass, i.e. either at its periphery and on top of the cell mass. Embryos formed at the periphery are in contact with the maturation medium and normally achieve a satisfactory quality in terms of morphology and accumulation of storage nutrients. Embryos formed on top of a proliferating cell mass are only in indirect contact with the maturation medium and are also affected by compounds (e.g. growth regulators) exudated or leaked from the proliferating cells. Often the consequence of this is delayed maturation, hyperhydricity, incomplete morphology (e.g. typically malformed or missing cotyledons), and decreased accumulation of storage nutrients.

Finally, it is known that the ability of established embryogenic cultures to undergo maturation declines with their age. For some species, especially the members of *Pinus* genus the decline is very rapid, i.e. within months. Cultures from several other genera such as *Picea*, *Larix* and *Abies* are more long-term stable, but in most cases, some sort of decline is observed either as a reduced maturation frequency or as a requirement for longer maturation periods or higher concentration of maturation agents such as abscisic acid.

Summary of the invention.

In a first aspect of the invention a method is provided for maturation of conifer somatic embryos, comprising a step, where an embryogenic cell mass is cultured with a culture medium comprising an anti-auxin.

By including an anti-auxin in the culture medium during at least part of the maturation, several unexpected and positive effects are obtained.

Proliferation is reduced. This means that the formation of new immature embryos ceases. Reduction of proliferation in itself facilitates the transition from proliferation to maturation. But maturation frequency is increased to a much larger extent than expected merely from the reduction in proliferation. The anti-auxin results in an unexpected shift in physiology from proliferation to maturation.

Almost all embryogenic cell lines tested, irrespective of the species, respond to the maturation treatment with production of mature somatic embryos. Thereby, a much higher maturation percentage than experienced by the prior art is obtained.

Surprisingly, it has been discovered that the quality of the somatic embryos is not reduced, although the activity of the important endogenous plant growth regulator, auxin, is reduced. As a matter of fact, the overall quality of the mature embryos harvested at the end of maturation is actually increased over the prior art.

The other components of the culture medium such as nutrients, vitamins, osmotica, organic nitrogen, gelling agent, carbon sources and plant growth regulators are not part of the inventive idea according to claim 1. The choice of the skilled person provides an infinite number of possibilities. The prior art contains numerous examples of suitable combinations of macro-nutrients and micro-elements (see e.g. George 1993) as well as suitable metabolisable carbon sources such as for instance sucrose, maltose, lactose, fructose, glucose, maltotriose, starch, galactose etc. In addition to these the person skilled in the art may chose to incorporate vitamins and various sources of organic nitrogen such as amino acids or complex mixtures of these such as casein hydrolysate or other hydrolysates in the culture medium. Furthermore it may be advantageous to include an osmoticum, such as a non-

permeating osmoticum such as for instance polyethylene glycols, dextrans, celluloses, pectins, galactans, ficolls, polypropylene glycols, agars, gums, oligosaccharides, proteins, amino acids, polyamino acids, lipoproteins, nucleotides, oligonucleotides, lipopolysaccharides, or permeating osmotic such as for instance polyethylene glycols, sugar alcohols, sorbitol, mannitol, and carbohydrates. The culture medium used during the various steps of maturation may be either liquid, the cells being cultured in the medium or in contact with the medium on some sort of solid phase support. Alternatively the medium may be gelled with one or more of the known gelling agents such as for instance gelrite, phytagel, agar, agarose, starch or similar agents. Irrespective of the culture medium, the cells may be cultured on a filter paper or similar support means, which facilitates subculture significantly.

Finally the culture medium used during maturation may comprise additional plant growth regulators such as cytokinins, gibberellins, or even auxins.

In the case, where one of the steps according to the invention is relatively short, e.g. less than 3 weeks, the culture medium may be very simple excluding one or all the groups of traditional medium components. The culture medium may also simply be water or gelled water, since the embryogenic cell mass may easily survive a period without nutrients and/or metabolisable carbon.

The length of the step comprising an anti-auxin is preferably between 2 days and 50 weeks, the length depending on the species and the specific cell line in question. Experiments have shown that whereas some cell lines should be cultured on medium with anti-auxin during the whole maturation period, other cell lines only require 2-4 weeks or even less to obtain the effects mentioned above. Similarly, the age of the cell line has a pronounced effect of the total length of the maturation period, younger cell lines such as less than one year old generally maturing much faster than old cell lines such as more than five years old. Thus, younger cell lines would normally also require a shorter period of exposure to anti-auxin. A sign of too long exposure to the anti-auxin is the appearance of malformed embryos with irregular or missing cotyledons. In this case the period of exposure to anti-auxin should be shortened.

Preferably the method further comprises a second step before the anti-auxin step, where the embryogenic cell mass is cultured with a culture medium. For the majority of species and cell lines, an exposure to anti-auxin right from the beginning of maturation has turned out to reduce the viability of the plated cells. This is partly due to the fact that newly plated cells are in a stressed condition and the culture needs to be "established" on the maturation medium before exposure to anti-auxin. The length of the second step before the anti-auxin step is advantageously between two days to 10 weeks.

Often it is likewise preferable to include a third step after the anti-auxin step where the embryogenic cell mass is cultured with a culture medium essentially free of anti-auxin. As mentioned above, a too long exposure to anti-auxin may have undesirable side-effects in which case the culture with maturing embryos has been transferred to a culture medium devoid of anti-auxin but including all other necessary components for continued maturation. Preferably the third step after the anti-auxin step lasts from two days to 40 weeks.

Unexpectedly, the shift of the addition of a metabolisable carbon source such as from maltose to sucrose in the culture medium in the third step omitting the anti-auxin gave a surprising positive maturation effect of the size of the mature embryos, the appearance of the embryos and shortening of the maturation period.

In a preferred embodiment of the invention, the culture medium in at least one of the steps also contains a maturation agent. Whereas embryo maturation is possible without the use of any maturation agent, it has been determined that the addition of such an agent to the culture medium increases the maturation frequency and the quality of the embryos greatly. Depending on the length of the various phases, the maturation agent may be present during one, two or all three steps. Preferably the agent is present during all three steps.

Preferably, the maturation agent is selected from the group comprising abscisic acid, silver nitrate, jasmonic acid, abscisyl alcohol, acetylenic aldehyde, dihydro-acetylenic alcohol, phaseic acid, dihydrophaseic acid, 6'-hydroxymethyl abscisic acid, beta-hydroxy abscisic acid, beta-methylglutaryl abscisic acid, beta-hydroxy-beta-methylglutarylhydroxy abscisic acid, 4'-desoxy abscisic acid, abscisic acid